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LETTER TO THE EDITOR

Re: Absence of Simian Virus 40 in Human Brain Tumors From Northern India; Response to Letter From Carbone *et al.*

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Dear Sir,

Carbone *et al.* advance a series of misstatements in criticizing our recently published study.[1] Contrary to their assertions, our study provides evidence that simian virus 40 (SV40) is unlikely to be a cause of brain tumors in northern India. We respond to their numbered comments in order.

1. Carbone *et al.* state that the low SV40 copy number amplified from the positive controls (formalin-fixed mouse tumors) indicates either that we added very little DNA to our PCR reaction mixture or that our assay was insensitive. However, the possibility that SV40 was inefficiently amplified due to the effects of formalin fixation was not considered by the correspondents.

As we write in our article, "The early phase of PCR amplification kinetics is susceptible to the DNA-degrading effects of formalin fixation, so the copy number derived from the growth curves for formalin-fixed tissues may not correspond to that for a similar amount of SV40 DNA present in the titration series."[1] Because of the effects of formalin fixation, one cannot directly extrapolate from an SV40 DNA titration series to estimate the number of SV40 copies present in a tumor sample. To address this problem, we simultaneously used quantitative PCRs for SV40 and a human gene, each standardized against an appropriate dilution series, to measure the amount of SV40 in tissue relative to cell content. Thus, as we write, we consider these results "semiquantitative."[1]

In this regard, we chose the mouse tumors as positive controls to mimic the conditions of archived human tissues, i.e., small amounts of formalin-fixed tissue. Under these conditions, filter hybridizations from all 7 SV40-positive mouse tumors were positive (Fig. 2 of our article). The quantitative PCR also detected SV40 DNA in 6 of these tumors. The low absolute level that we measured (equivalent to $<$ 10 copies of SV40 per reaction) likely arose from a combination of low tissue input and the effects of formalin fixation. Rather than indicate an insensitivity of our assay, as Carbone *et al.* claim, the published results demonstrate that, even in such challenging circumstances, our quantitative PCR is highly sensitive in detecting SV40.

2. In their second criticism, the logic of Carbone *et al.* is less clear. They apparently acknowledge that formalin fixation of tissues decreases the quality of DNA and leads to a lower quantitative PCR estimate of SV40 copy, as we indicate above. The correspondents then suggest that the DNA-degrading effects of fixation might require more than 50 cycles of PCR to detect SV40 in positive tumors. However, using only 50 PCR cycles, we consistently identified SV40-positive mouse tumors using both quantitative PCR and filter hybridization. Also, as we discuss next, the simultaneous PCR-based quantification of a human gene indicated that, despite the technical difficulties created by the formalin fixation, adequate amplifiable DNA was present in our experiment.

3. As mentioned, we standardized the measured SV40 copy number to the number of detected copies of a human cellular gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH). In our article, we mistakenly refer to this enzyme as glyceraldehyde-6-phospate dehydrogenase, and we thank the correspondents for correcting our typographical error. More substantively, Carbone *et al.* suggest that the presence of GAPDH pseudogenes could have caused us to overestimate the number of cell-equivalents of amplifiable DNA present in our reaction mixtures. We admit that we had not adequately considered this possibility. Based on a 1984 article, Carbone *et al.* point to the possible existence of 25 copies of GAPDH-"homologous" sequences in the human genome.[2] However, by BLAST search, we identified only 4 known sequences in the human genome that matched our primers and that would result in amplification of the observed product size.

The uncertainty raised by the limited data on this question prompted us to compare results obtained using our GAPDH quantitative PCR with those from a similar quantitative PCR assay for ERV-3,[3] an endogenous retrovirus present in 1 copy per haploid genome. In duplicate, we prepared 60 aliquots of DNA extracted from Ramos cells, corresponding to approximately 50 to 4,000 cells per aliquot. These aliquots were assayed independently in separate laboratories for GAPDH and ERV-3 copy number. The GAPDH copy number was consistently greater than the ERV-3 copy number (median 9,300, range 700–99,000, *vs.* 1,200, range 120–8,300). The median GAPDH/ERV-3 ratio was 7.0, generally consistent with the results of the BLAST search.

These observations lead us to recalculate 2 results from our study.[1] First, in the single ependymoma that provided an unconfirmed positive result, we now estimate that SV40 was present in at most 1 copy per 50 cells (i.e., $<$ 10 copies/[7,009 GAPDH copies/14 GAPDH copies per cell]). Second, based on

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the observed GAPDH copy numbers, 50% of our evaluated tumor specimens had at least 105 cell-equivalents of amplifiable DNA (i.e., median GAPDH copies 1,476/14 GAPDH copies per cell). None of the tumors had detectable SV40. Thus, the experiment ruled out SV40 at a level of more than 1 copy per 10 cells (i.e., 10 copies/105 cells). These 2 negative results, coupled with the negative filter hybridization,[1] remain convincing arguments against the presence of SV40 in these tumors.

Two additional comments by the correspondents deserve a brief response. They point out that K562 cells are triploid. However, we used K562 cells only as a ready source of human DNA for PCR experiments. Specifically, the GAPDH copy number in DNA extracted from these cells was standardized to a known concentration of a plasmid containing a single copy of GAPDH. Therefore, the ploidy of the source of DNA used in the standard is not relevant. Carbone *et al.* also argue that only a portion of our specimens consisted of tumor. However, our pathologic review of tissue sections adjacent to those evaluated by PCR indicated that all of the specimens were comprised predominantly of tumor cells.

4. Carbone *et al.* mistakenly write that we suggest that our assay could have detected 10 copies of SV40 in fixed tissue specimens. As stated already, we acknowledge that formalin fixation reduces the sensitivity of PCR. Thus, we do not believe that a measured amount of SV40 DNA amplified from formalin-fixed tissue would correspond to the amount that would have been amplifiable in the same tissue when fresh. However, because formalin fixation likely affects SV40 and GAPDH amplification equally, the absence of quantifiable SV40 DNA, when readily measurable GAPDH was present, is strong negative evidence. In coupling the SV40 and GAPDH PCR results, we rule out SV40 at a level of 10 or more copies per 105 cells.

5. In a final criticism, Carbone *et al.* claim that our study was not truly masked, based on an examination of our Figure 2, which shows the titration series and water controls placed consecutively. However, this assertion reflects another misunderstanding, because it was only the human tissues (brain tumor cases and other brain tissues that served as negative controls) that were masked and sorted in a random order. For example, in Figure 2 of our study,[1] tissues in row A (columns 3–12) correspond to: tumor, normal, tumor, tumor, tumor, tumor, normal, tumor, normal, tumor. Because these tissues were randomly sorted and masked before extraction and PCR testing, our experiment is an appropriate test of whether SV40 is preferentially present in human tumors over tissues from other, presumably SV40-unrelated, brain conditions.

Carbone *et al* argue that the sensitivity of our methods was limited. While acknowledging the challenges in working with archival tumor tissue, we have presented evidence that our experiment had adequate sensitivity to effectively rule out the presence of SV40 in Indian brain tumors. It is appropriate, then, to briefly highlight our concerns regarding published studies that have reported the detection of SV40 in brain tumors (see Table 1 of our article).[1] Again we point out that an important weakness in these studies was that many lacked a control series of human tissues in which SV40 was expected, *a priori*, not to be present. Also, none of the studies evaluated tissues in a manner masked to tissue status (tumor *vs.* control). These limitations call into question the specificity of SV40 detection, which has been reported variably for diverse tumors (including but not limited to various brain tumors, sarcomas, mesothelioma, lymphoma) and normal tissues. This concern could be addressed rather simply, by including negative control tissues, evaluated in a masked manner concurrently with tumors of interest, analogous to our experiment. Importantly, in a masked multiinstitutional study evaluating mesothelioma specimens, the frequency of detection of SV40 sequences was very low and identical to that in masked controls.[4]

As we indicate, the quantification of SV40 DNA in evaluated tissue specimens, while rarely undertaken, can provide further relevant information regarding biologic plausibility.[1] In their letter, Carbone *et al.* suggest that Southern blots can provide an approximate quantification of the amount of SV40 DNA present in cell lines. If this approach is feasible, it would be important to examine similar data for mesothelioma or brain tumor specimens obtained from humans. Of interest, Gorden *et al.* recently used a quantitative PCR assay similar to ours to detect and quantify SV40 DNA in U.S. mesothelioma specimens.[5] Notably, SV40 DNA was present in only 2 specimens (6%), at a level of less than 1 copy per 134 cells. Overall, the suggested presence of SV40 DNA in a wide range of tumors and normal tissues, detected variably across laboratories and in at most extremely low levels, calls reported associations into question.

We recognize the heated controversy surrounding the question of SV40 in human tumors. Therefore, it was inappropriate for the correspondents to suggest that there is resolution or consensus on this matter within the scientific community. Carbone *et al.* distort the record when they imply that the Institute of Medicine report concluded that there is compelling evidence in support of an association between SV40 and cancer. That report noted that "The conflicting results in the detection of SV40 have also led to questions about technical aspects of the detection of the virus. It is unclear whether positive findings are the result of overly sensitive but nonspecific tests that are detecting other viruses (i.e., BK or JC) or SV40 from laboratory contamination . . ."[6] Carbone *et al.* also distort the record in their summary of a published commentary on a recent National Cancer Institute workshop.[7] In that commentary, Wong *et al.* wrote "Many epidemiologists and some laboratory investigators remain unconvinced of the validity or consistency of detection of SV40 in human tumors or the significance of the various reported detections."[7] Given widespread doubt among scientists, it then seems remarkable that the "international consensus" meeting, organized by Dr. Carbone and colleagues in 2001, could have reached the conclusion that "there is now overwhelming evidence proving that SV40 is capable of infecting humans and that SV40 is present in some mesotheliomas . . ."[8] Absent consensus, we agree with Wong *et al.* that "More research is needed to resolve the controversial issues . . . of whether SV40 is involved in human cancers."[7] We urge that future laboratory studies of SV40 in human cancers be conducted using rigorous designs that incorporate appropriate negative controls and masking.

Yours sincerely,

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